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POUNDS AS ESSENTIAL GROWTH
SUBSTANCES FOR DYSENTERY
BACILLI

AN EXPANSION OF DISSERTATION SUBMITTED
TO THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY


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By
ALBERT DORFMAN

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NICOTINAMIDE AND RELATED COMPOUNDS AS ESSENTIAL GROWTH SUBSTANCES FOR DYSENTERY BACILLI

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In previous communications it was shown that certain fractions of veal infusion and of extracts of spleen, liver and other tissues supplied a growth factor needed by several strains of dysentery bacilli for development in a synthetic medium.¹ It was later found that the addition of nicotinic acid or its amide to the synthetic medium caused prompt growth of the dysentery bacilli.² The growth-promoting activity of certain compounds related to nicotinic acid was also reported in preliminary form.³

It is the intention in this paper to report the isolation of nicotinic acid and its amide from spleen, together with a more detailed discussion of the activity of nicotinic acid and related compounds on a larger number of strains of the dysentery bacillus.

METHOD OF ASSAY

The growth-promoting activity of unknown preparations was measured by the addition of small amounts of the preparation to a basal synthetic medium which was otherwise incapable of supporting growth. Routine assays were done on at least three dilutions of each preparation, with a more complete series of dilutions for those preparations of greater significance. In this way the minimum quantity of the preparation which would support growth was determined.

The basal medium used in this investigation is given below.

Synthetic Medium

Dipotassium hydrogen phosphate.....	1.0 gm.
Magnesium sulfate.....	0.1 gm.
l (+)—glutamic acid.....	0.5 gm.
l (+)—alpha alanine.....	0.5 gm.
glycine.....	0.2 gm.
l (+)—lysine. 2 HCl.....	0.2 gm.
l (—)—tryptophane.....	0.2 gm.
l (—)—histidine. 2 HCl.....	0.2 gm.
dl --valine.....	0.1 gm.

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1. Koser, S. A., and Saunders, F.: J. Infect. Dis. **56**: 305, 1935; **58**: 121, 1936.

2. Koser, S. A., Dorfman, A., and Saunders, F.: Proc. Soc. Exper. Biol. & Med. **38**: 311, 1938.

3. Dorfman, A., Koser, S. A. and Saunders, F.: J. Am. Chem. Soc. **60**: 2004, 1938.

<i>l</i> (-)—leucine.....	0.1 gm.
<i>dl</i> —phenyl alanine.....	0.1 gm.
<i>l</i> (-)—cystine.....	0.1 gm.
<i>l</i> (-)—proline.....	0.1 gm.
<i>l</i> (-)—hydroxyproline.....	0.1 gm.
<i>dl</i> —methionine.....	0.1 gm.
<i>l</i> (+)—arginine. HCl.....	0.1 gm.
<i>l</i> (-)—tyrosine.....	0.05 gm.
Redistilled water.....	1000 cc.

The p_H is adjusted to 6.8–7.0 with normal sodium hydroxide solution. The medium is then tubed in 5 cc. quantities and after sterilization in the autoclave enough sterile glucose solution is added to make a final glucose concentration of 0.2%.

In connection with the use of synthetic mediums in studies of this kind it must be remembered that little is known of the amino acid requirements of micro-organisms. Studies of the amino acid requirements of the more fastidious micro-organisms have always required the addition of certain tissue extracts in order to furnish essential growth substances. Since these extracts were not usually free of amino acids, it has been impossible in most cases to obtain exact information of the amino acid requirements.

The amino acid requirements of the dysentery bacilli have not been studied in detail. There is some evidence that the 15 amino acids in the synthetic medium supplied a greater variety than was actually needed by many of the dysentery strains. On substitution of a simpler medium containing asparagine, tryptophane and cystine, some of the dysentery bacilli developed as readily in the presence of adequate amounts of growth factor as in the more complex medium. Others were unable to grow in the simpler medium and the amino acid requirements of these cultures were not investigated further at this time. In a collection of 33 cultures three were found which were able to develop promptly when an ammonium salt was substituted for the amino acids.

Inoculations into the synthetic medium were always made by adding 0.1 cc. of a very light suspension of the organism in a buffered salt solution. This suspension was made just before use by inoculating very lightly from a 24-hour culture on nutrient agar. This procedure served to give all tubes of any one series approximately equal inoculations and avoided carrying over of appreciable amounts of growth factors in the inoculum.

Growth was estimated by the visual determination of turbidity and was recorded as negative, one, two or three plus. We have also used the titration method in certain studies. A more detailed discussion of this method will be presented in a later section of this paper.

ISOLATION OF THE GROWTH FACTOR FOR THE DYSENTERY BACILLUS FROM PIG SPLEEN

It was found by Koser and Saunders¹ that the active principle could be extracted from various tissues by hot water. Finkle⁴ showed that 20–40% of the inert organic solids in a spleen extract could be removed by precipitation

4. Unpublished Ph.D. dissertation, Department of Biochemistry, University of Chicago, 1937.

with neutral lead acetate. He further showed that if sufficient ammonium hydroxide was added to the first lead filtrate to bring the solution to p_H 8-9 a voluminous precipitate was formed which removed another 10-15% of the inert material.

Preparation of crude extracts and preliminary purification.—Pig spleen was used as a starting material, since it was found to be as rich a source as liver and is much cheaper. The spleen was ground in a meat grinder, covered with water and set in the refrigerator for about 48 hours. Then the spleen was boiled for about 5 minutes and the first extract was poured through a forty-mesh strainer. The coagulated tissue was again covered with water and the mixture boiled for 5 minutes. The combined extracts were heated to boiling and a boiling solution of neutral lead acetate was added until no further precipitation occurred. The heavy precipitate which formed settled rapidly and a slight excess of lead acetate was added. The precipitate was filtered off, a small quantity of fuller's earth being added to aid the filtration. Fuller's earth does not adsorb the activity. The filtrate obtained was a clear, light-yellow solution which became cloudy on cooling. The solution was concentrated in vacuo so that a liter of solution was equivalent to 5 pounds of spleen.

To each 10 liters of solution was added 1 liter of concentrated ammonium hydroxide (sp. gr. 0.90). This gave rise to a voluminous precipitate which was found to be inactive. The precipitate was filtered off and a light-yellow filtrate was obtained. This filtrate was then concentrated on the steam bath or in vacuo to a point where 1 liter of solution was equivalent to 35 pounds of the original tissue. As the solution was concentrated a small amount of inert, amorphous material separated.

The concentrated material was then treated with hydrogen sulfide to remove the lead. The precipitation of the lead sulfide removed inert material without affecting the activity. The filtrate from the lead sulfide was further concentrated on the steam bath.

On cooling the solution in the refrigerator a heavy brown precipitate formed. The precipitate carried down considerable activity but the activity could be washed off the precipitate⁵ with ethanol. The ethanol washings were combined with the filtrate. The material thus prepared contained all of the activity present in the original infusion.

Benzene extractions.—A number of extractions were made at different hydrogen ion concentrations to determine the optimum p_H for extraction of the activity. The extractions were carried out in a Dakin type continuous liquid extractor. The material prepared as indicated above was at a p_H of about 5.5 as determined by the glass electrode. It was found that at this p_H about 75% of the activity was extracted in the first 24-hour period, and that on continued extraction a small amount of additional activity was extracted each day. However, we were never able completely to extract the activity by the use of benzene at this p_H . Table 1 shows the relative activity of the

5. This precipitate is composed principally of amino acids of which leucine makes up the largest part. The quantity of this precipitate varied greatly in different preparations.

benzene and water phases after extraction for 4 days. The rate of extraction was not measured in this case, but it was found that extracting at the rate of 120 cc. per minute for 2 days did not result in complete extraction. If after a 3 or 4 day extraction with benzene at a p_H of 5-6, the solution is made alkaline with sodium hydroxide to a p_H of about 11.0, another large portion of activity can be extracted.

These facts led us to believe that our solutions must contain more than one active compound. Extractions of pure solutions of nicotinic acid and of nicotinamide gave results which indicated that we were dealing with a mixture of these two compounds. The failure to obtain complete extraction at an alkaline pH may be due to the slow hydrolysis of the amide at such a p_H . The extracts obtained at an acid p_H were investigated more thoroughly and will be discussed in greater detail.

TABLE 1.—*Extraction of Activity by Benzene at a p_H of 5.5*

Preparation	Amount of Extract Added to 5 cc. of Synthetic Medium cc.	Growth of Dysentery Bacilli, Strains 76 and 74, at 37 C.							
		76 days				74 days			
		1	2	4	10	1	2	4	10
Original material	0.001	+++	+++	+++	+++	+++	+++	+++	+++
	0.0003	+++	+++	+++	+++	+++	+++	+++	+++
	0.0001	+	+	+	+	0	0	0	0
	0.00003	0	0	0	0	0	0	0	0
	None	0	0	0	0	0	0	0	0
Benzene extract	0.001	+++	+++	+++	+++	+++	+++	+++	+++
	0.0003	+++	+++	+++	+++	+++	+++	+++	+++
	0.0001	0	0	0	0	0	0	0	0
	0.00003	0	0	0	0	0	0	0	0
	0.00001	0	0	0	0	0	0	0	0
Benzene extracted water phase	0.001	+	+	+	++	+	++	++	+++
	0.0003	0	0	+	+	0	0	0	0
	0.0001	0	0	0	0	0	0	0	0
	0.00003	0	0	0	0	0	0	0	0
	0.00001	0	0	0	0	0	0	0	0
	None	0	0	0	0	0	0	0	0

0, no visible growth

+, very light turbidity just at point of visibility

++ to +++, light to very pronounced turbidity

Isolation and identification of nicotinic acid.—The benzene extracts representing 600 pounds of spleen were combined and were taken to dryness in vacuo. The residue was taken up in about 500 cc. of ethanol. This solution was divided into two different fractions for high vacuum distillation. Both were treated in the same manner, and were recombined at a later stage.

On a trial run it was found that all the activity in these extracts could be distilled in high vacuum at a temperature of 100-150 C. with a pressure of 5×10^{-5} mm. of mercury. The temperature of the retort was kept relatively constant by heating in an air bath within an electrically heated cast aluminum jacket.

Distillations were continued for from 72 to 96 hours. At least three distinct fractions were observed in the distillate. The first of these was a yellow oil which collected in the U tube immersed in a carbon dioxide-acetone bath. This fraction was inactive and was not investigated further. The second and third fractions were composed of less volatile material which collected im-

mediately beyond the boundary of the heating unit. One of these was a yellow viscous oil, while the other consisted of a mass of white crystals.

The retort was cut to separate the residue from the distillate and a large part of the oil was poured out. The remaining oil and mass of crystalline material were washed from the retort with ethanol. The ethanol washings from two separate runs on material equivalent to a total of 600 pounds of tissue were combined.

On concentration to 30 cc. and cooling in the refrigerator, a precipitate separated. This material was crystalline under the microscope. The crystals (about 20 mg.) were not uniform. They were soluble in water, ethanol and methanol, but insoluble in ether and benzene. Heating up to 275 C. in a sealed capillary tube resulted in no decomposition or melting, but sublimation could be noted. The crystals were then recrystallized from 10 cc. of methanol. The recrystallized material was inactive. An attempt to determine the melting point of the crystalline fraction by means of an open electrically heated stage resulted in sublimation from 220–40 C. On microscopic examination two distinct crystal types could be seen in the sublimate. One compound crystallized in irregular plates (a few regular hexagons were seen), while the other type consisted of small prisms. The components of this mixture were not identified.

The ethanol filtrate from the crystals discussed above was combined with the methanol mother liquors. This combined fraction was concentrated to 10 cc. and more crystalline material separated on cooling. The quantity was too small to be filtered but appeared microscopically similar to the prisms seen above.

Fifty cc. of ether was added to the alcohol and a yellowish brown precipitate was thrown down. The ether-alcohol phase was decanted and the precipitate was washed twice with ether. The precipitate was then extracted 5 times with 5 cc. portions of boiling benzene. A large portion of the precipitate thrown down by ether was insoluble in benzene. The fractionation of this material will be discussed later.

The benzene solution was concentrated to 1 cc. and set in the refrigerator. A small amount of crystalline material appeared on the sides of the flask. This appeared to consist of a mesh of fine needles when examined microscopically. The quantity of material obtained was not sufficient to permit purification. After washing a small amount several times with ether, the melting point was determined on an electrically heated hot stage. The material melted over a range of 120–30 C. Since the melting point of nicotinamide (m.p. 125 C.) is in this range, it was decided to attempt to prepare an insoluble derivative. The picrolonate of nicotinamide has been described by Warburg.⁶ It is insoluble in cold water and melts with decomposition at 215 C.

The benzene was removed from the above-mentioned solution and the residue was dissolved in 2 cc. of water. One cc. of a saturated solution of picrolonic acid in water was added and the mixture boiled for a short time. On cooling the solution in the refrigerator a flocculent, yellow precipitate

6. *Biochem. Ztschr.* **182**: 157, 1935.

appeared. This was filtered off, the quantity obtained being very small. The melting point as determined on an electrically heated hot stage was 175–85 C. The compound appeared to decompose on melting. A sample of picrolonate of nicotinamide prepared in a similar manner gave a melting point of 190–5 C. A mixture of the picrolonate prepared from the known and that prepared from the unknown gave a melting point of 175–90 C.

The picrolonate prepared from the unknown was recrystallized once from 1 cc. of water. After recrystallization this compound melted more sharply at 203–5 C. A mixture of this preparation and a similar preparation of nicotinamide melted at 202–6 C. Further work on this fraction had to be abandoned due to the extremely small amount that remained at this stage.

The results obtained above indicate that this fraction was probably made up of nicotinamide, but the small amount of material obtained made absolute identification impossible. In the first part of this section it was stated that nicotinamide is only slowly extracted by benzene at a p_H of 5.5. It is, therefore, not surprising that we obtained such small quantities of this compound.

The part of the precipitate that is thrown down by ether and is insoluble in benzene was dissolved in 5 cc. of boiling ethanol. On cooling in the refrigerator a crystalline precipitate separated out, but the quantity obtained was too small for further work. This compound sublimed between 160 and 180 C.

The alcohol solution was diluted with 20 cc. of water and 3 cc. of a 5% cupric acetate solution was added. On boiling, a light blue-green precipitate formed. This precipitate was microscopically identical with the copper salt of nicotinic acid. The precipitate was filtered off and suspended in 25 cc. of water. Hydrogen sulfide was passed into the solution with continuous agitation. The copper sulfide was filtered off and a water clear solution was obtained, which was found to be comparable to nicotinic acid in growth-promoting activity for dysentery bacilli. This solution gave a purple-red color with chloro-2, 4-dinitrobenzene. This color reaction is typical of nicotinic acid.⁷ On concentration to about 0.5 cc. this solution gave a crystalline compound which sublimed at 170–80 C. on an electrically heated hot stage. Since nicotinic acid is quite soluble in water, it was decided to reprecipitate the unknown material as the copper salt which is very insoluble.

The copper salt was reprecipitated as described above. A blue precipitate which weighed 65 mg. was obtained. This salt was identified as the copper salt of nicotinic acid by analysis; for Cu, calcd. 31.38%, found 31.45%, for N, calcd. 6.91%, found 7.18%.

The alkaline extracts have not been investigated as thoroughly as those described above, but work has been done which suggests the presence of nicotinamide and absence of nicotinic acid in these preparations. High vacuum distillation of these preparations yielded fractions similar in appearance to those previously described. It was found that the activity could be quantitatively distilled from these preparations but that the distillates failed

7. Vilter, S. P., Spies, T. D., and Mathews, A. P., *J. Biol. Chem.* **125**: 85, 1938.

to give a copper salt. Sufficient material was not available to attempt isolation of the active compound in these fractions.

Nicotinic acid has been isolated from pig spleen and the presence of the amide is strongly indicated. The acid does not necessarily occur free in the original spleen but may have been formed from the amide or from more complex materials in the process of isolation.

NICOTINAMIDE AND COENZYMES I AND II AS GROWTH FACTORS
FOR DYSENTERY BACILLI

Nicotinic acid and its amide have been known chemically for many years as derivatives of the alkaloids. Suzuki⁸ isolated nicotinic acid from rice polishings in 1912. Funk⁹ isolated impure nicotinic acid from yeast and rice bran, but when this failed to cure the symptoms of a combined vitamin B complex deficiency he dropped further investigation of this compound.

More recently Warburg⁶ showed that nicotinamide was a part of the coenzyme necessary for the oxidation of hexose monophosphate by the "Zwischenferment" of erythrocytes. Euler¹⁰ about the same time showed that cozymase, a coenzyme present in yeast, was a diphosphopyridine nucleotide consisting of nicotinamide, adenine and *d*-ribose.

Knight¹¹ found that nicotinic acid and its amide together with thiamin would promote the growth of *Staphylococcus aureus* in a synthetic medium. Mueller^{12,13} showed that nicotinic acid together with β -alanine were essential for the growth of the diphtheria bacillus.

Elvehjem and his coworkers discovered that nicotinamide or nicotinic acid was able to cure canine blacktongue.¹⁴ These investigators were able to isolate nicotinamide from liver concentrates. On the basis of the long-suspected similarity between canine blacktongue and human pellagra, Spies,¹⁵ Smith¹⁶ and Fouts et al.¹⁷ fed nicotinic acid to patients suffering from pellagra. They found that nicotinic acid or nicotinamide was able to cure all of the symptoms of pellagra that were not associated with other deficiencies. Another report indicated that nicotinic acid was also essential for the nutritional well-being of pigs.¹⁸ A recent paper by Addicott and Bonner¹⁹ showing that nicotinic acid promoted the growth of pea roots is of special interest. Nicotinic acid is apparently significant in the metabolism of higher plants.

During the course of the work on the isolation of nicotinic acid from spleen, the related compounds, nicotinamide and coenzymes I and II were

8. Biochem. Ztschr. **43**: 89, 1912.

9. J. Physiol. **47**: 173, 1913.

10. Ztschr. f. physiol. Chem. **237**: 1, 1935.

11. Biochem. J. **31**: 731, 1937.

12. J. Bact. **34**: 381, 1937.

13. J. Bact. **34**: 429, 1937.

14. J. Biol. Chem. **123**: 137, 1938.

15. J.A.M.A. **110**: 622, 1938.

16. J.A.M.A. **109**: 2054, 1937.

17. Proc. Soc. Exper. Biol. & Med. **37**: 405, 1937.

18. Chick, H., et al.: Biochem. J. **32**: 10, 1938.

19. Science **88**: 577, 1938.

tested as growth factors for the dysentery bacilli. For this purpose a collection of cultures was assembled so as to include several representatives of each of the principal subgroups of the *Shigella* genus.

Thirty-three cultures were tested for their ability to develop in a synthetic medium alone and with the addition of either nicotinic acid, nicotinamide, coenzyme I or coenzyme II.²⁰ Sterile filtered solutions of these compounds were added aseptically to the synthetic medium. Nicotinic acid and nicotinamide were sterilized by autoclaving. Most of the dysentery cultures were stock strains which had been kept on the usual laboratory culture mediums for several years or longer. A number of them were from our own laboratory collection, while others were secured from other laboratories.²¹

The results are summarized in table 2. Of 18 members of the Flexner subgroup, 14 were unable to grow in the synthetic medium, but did develop

TABLE 2.—*The Effect Upon Development of Dysentery Bacilli of Nicotinic Acid, Nicotinamide and Coenzyme When Added to the Synthetic Medium*

Dysentery Subgroup and Number of Cultures	Synthetic Medium Only	Synthetic Medium with Added			
		Nicotinic Acid	Nicotinamide	Coenzyme I	Coenzyme II
Flexner { 11 cultures	0	+++	+++	+++	+++
{ 3 cultures	0	+++*	+++*	+++*	+++*
{ 4 cultures	+++	+++	+++	+++	+++
Shiga—6 cultures	+++†	+++	+++	+++	+++
Sonne—6 cultures	— or +	+++	+++	+++	+++
<i>Shigella alcalescens</i> —3 cultures	+++	+++	+++	+++	+++

The results shown in the table are for 24 hours at 37 C., unless otherwise stated. The amount of added nicotinic acid and nicotinamide was 0.25 microgram per cc. of medium; of coenzymes I and II 1.0 microgram per cc. of medium.

* Slow development, with visible turbidity appearing in from 3 to 7 days.

† Attained +++ growth usually within 24 to 48 hours rather than at 24 hours.

upon the addition of nicotinic acid, nicotinamide, coenzyme I or coenzyme II. Eleven of these 14 cultures developed readily, showing pronounced turbidity within 24 hours, while the other 3 developed more slowly. The remaining 4 cultures of the Flexner subgroup developed in the synthetic medium without added accessory material. Six representatives of the Shiga type were all able to multiply in the synthetic medium, though growth often appeared sooner upon the addition of nicotinic acid or one of the related compounds. Six cultures of the Sonne type were unable to develop or developed only to the extent of a very slight turbidity in the synthetic medium. These cultures developed readily upon the addition of one of the accessory substances. Three strains of *Shigella alcalescens* all developed readily in the synthetic medium without added factors.

Other known growth factors were not capable of replacing the coenzyme or its pyridine component in supplying the requirements of dysentery bacilli. Thus riboflavin, thiamin, β -alanine and inositol, when added to the synthetic medium either singly or in combination failed to support growth. When

20 We are indebted to Dr. A. Axelrod of the University of Wisconsin for the sample of coenzyme I which had been prepared in Euler's laboratory and to Dr. Otto Warburg for the supply of coenzyme II.

21. For the cultures we are indebted to Dr. W. J. Nungester of the University of Michigan, Dr. Erwin Neter of Buffalo and to the New York State Department of Health Laboratories.

riboflavin, pantothenic acid, β -alanine, thiamin, inositol, pyrophosphate and Hoagland's salt mixture were all added in addition to either nicotinic acid or nicotinamide no increase in either growth or acid production was detected.

The development of the 3 cultures of the Flexner subgroup which grew slowly on addition of nicotinic acid, nicotinamide or the coenzymes (table 2) was not improved by the addition of riboflavin, thiamin, β -alanine, pyrophosphate and the amino acids threonine and serine.

Comparison of growth of representative cultures in the synthetic-nicotinic acid medium and in veal infusion broth showed that cell multiplication took place more rapidly in the latter medium. This could be demonstrated readily by observing cultures during the earlier stages of development. In tubes which had received equal inoculations, turbidity always appeared in the veal infusion before it became evident in the synthetic-nicotinic acid medium. After an interval of 10 to 12 hours at 37 C. growth in the veal infusion medium was quite pronounced while in the synthetic medium a light turbidity was often just apparent. However, after 24 hours at 37 C. the luxuriance of growth of many of the cultures in the synthetic medium approached more closely that in the veal infusion broth.

Successive transplants.—To determine whether those cultures which required nicotinic acid or the amide were able to develop continuously when these compounds were supplied, 8 representative cultures were subjected to successive transplants in synthetic medium containing 0.5 microgram per cc. of either nicotinic acid or nicotinamide. All tubes were inoculated originally with 0.1 cc. of a light suspension of cells in a buffered salt solution. Subsequent inoculations from tube to tube were made every 24 hours with a loop. One lot of 3 cultures was carried without any apparent diminution in the rate or luxuriance of growth through ten successive transplants in the synthetic medium plus nicotinic acid. Another series of 5 cultures was carried in a similar manner through 20 successive transfers in the same medium with nicotinamide.

Effect of increased amounts of nicotinic acid.—To determine whether relatively high concentrations of nicotinic acid would inhibit development, varying amounts of the acid were added to the synthetic medium to produce final concentrations of 1, 10, 100, 1,000, 3,000, 5,000 and 10,000 micrograms per cc. of medium. With the larger amounts it was necessary to adjust the pH to the original value of 6.8–7.0. Sterile dextrose solution was added to give a 0.2% solution of the sugar. Five cultures were used which were known to develop promptly upon addition of 0.5 or 1.0 microgram of nicotinic acid per cc. of medium.

The method used to detect any restraining effect upon development was that of recording the average time required for first appearance of visible turbidity, together with a comparison of the relative turbidities shown in different concentrations, especially during the interval of 10 to 24 hours after inoculation. With the amount of inoculum used rarely did visible growth appear in less than ten hours.

Amounts up to 1,000 micrograms per cc. of medium seemed to have little or no effect upon the speed of development, visible growth appearing at

about the same time in all cases. Also, the rate of subsequent development of the cultures appeared to be parallel. Above 1,000 micrograms per cc. growth was definitely affected as may be seen from the results given in table 3. Development of 2 of the 3 cultures was slightly retarded in the presence of 3,000 micrograms, very definitely retarded with 5,000 micrograms and completely inhibited by 10,000 micrograms. Thus, large amounts of nicotinic acid may be tolerated before a toxic effect becomes apparent. Since from 0.2 to 0.4 microgram per cc. is capable of supporting optimum growth of most dysentery cultures in the synthetic medium, it is evident that amounts up to approximately three thousand times this concentration do not restrict growth to any appreciable extent.

Relative activity of acid and amide.—The effect of a limited supply of nicotinic acid and nicotinamide was next studied. To 5 cc. amounts of the

TABLE 3.—*Inhibition of Growth of Dysentery Bacilli Produced by Large Amounts of Nicotinic Acid*

Culture No.	Synthetic Medium With Added Nicotinic Acid, Micrograms per cc.	Development of Culture as Shown by Visible Turbidity Hours After Inoculation					
		12	15	18	23	34	48
74	1000	+	+	++	+++		
	3000	0	+	+	++	+++	
	5000	0	0	0	+	++	+++
	10000	0	0	0	0	0	0*
76	1000	0	0	+	+	+++	
	3000	0	0	+	+	+++	
	5000	0	0	0	0	+	++
	10000	0	0	0	0	0	0*
3	1000	++	+++				
	3000	++	+++				
	5000	+	++	+++			
	10000	0	0	+	+	++	++

The readings shown are the average of triplicate tubes. Usually all three tubes of a series were the same.

* Remained negative through 10 days of observation.

synthetic medium graded quantities of the test substances were added to give concentrations ranging from 0.4 to 0.001 microgram per cc. of medium. After addition of the test substance all tubes were inoculated with 0.1 cc. of a light suspension of the organism. The inoculated tubes were incubated at 37 C. and observed at frequent intervals. Table 4 shows a representative assay of nicotinic acid and nicotinamide on one strain of the dysentery bacillus. It will be noted that on the first day the amide appears to be at least ten times as active as the acid. This difference is due to a slow development of the culture when small amounts of the acid are supplied. This does not occur when the amide is used; the cultures attain their maximum turbidity within 48 hours.

This phenomenon was checked further by the use of a more sensitive method. Cultures were started in the synthetic medium containing 0.5% glucose. Dilutions of nicotinamide and nicotinic acid were added under sterile conditions and the tubes inoculated in the usual way. After various intervals the cultures were titrated with N/100 sodium hydroxide, 0.3 cc. of 0.0008 M brom thymol blue having been added to each tube previous to titration. The cultures were titrated to the blue color of the indicator; the end point was determined by comparison with a standard color tube.

Figure 1 indicates the titration values as plotted against time. Each point is an average of 3 titrations after blank correction. It will be noted that the titration value seldom rises above 2.4 cc. N/100 sodium hydroxide. This value corresponds to a pH of 4.7 which apparently inhibits further growth.

The increase in titer with time is much slower in tubes containing the acid than in those containing the amide. All of the tubes containing the amide reach a figure close to their maximum at the end of two days while the acid tubes continue to increase in acidity up to the fourth day. In a few experiments in which titration values were determined on the seventh day the tubes containing small amounts of nicotinic acid showed a further increase in acidity. The final values in each case are substantially the same. Any comparison of activity of nicotinamide and nicotinic acid must take into account this difference in the rate of development.

TABLE 4.—Comparison of Growth-Promoting Activity of Nicotinic Acid and Nicotinamide for *Shigella paradysenteriae* Flexner

Micrograms per cc. of Medium	Nicotinic Acid					Nicotinamide				
	1	2	3	4	7	1	2	3	4	7
0.1	+++	+++	+++	+++	+++					
0.08	+++	+++	+++	+++	+++					
0.06	+++	+++	+++	+++	+++					
0.04	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
0.02	0	++	+++	+++	+++	+++	+++	+++	+++	+++
0.01	0	0	++	+++	+++	+++	+++	+++	+++	+++
0.008	0	0	+	+++	+++	+++	+++	+++	+++	+++
0.006	0	0	?	+	+++	+++	+++	+++	+++	+++
0.004	0	0	0	?	++	++	++	++	++	++
0.002	0	0	0	0	0*	+	+	+	+	+
0.001	0	0	0	0	0*	+	+	+	+	+
0.0008						?	?	?	+	+
0.0006						0	?	?	0	0
0.0004						0	0	0	0	0
None	0	0	0	0	0	0	0	0	0	0

* Growth occasionally appeared upon continued incubation up to 10 or 14 days.

These results indicate that the acid may be converted to the amide before it is utilized by the organism. This would be expected since the pyridine component of the coenzymes is nicotinamide. Knight²² has assumed such a mechanism in his interpretation of the necessity of nicotinamide for the growth of *Staphylococcus aureus*. Comparison of the two compounds is also of interest in connection with reports concerning other micro-organisms. Knight¹¹ found that the amide was about five times as active as nicotinic acid in supporting growth of the staphylococci. The results in his table are for twenty-four hours. The opposite effect was reported by Mueller.¹³ He found that nicotinamide is only about one-tenth as effective as is nicotinic acid in supporting growth of the diphtheria bacillus.

Many writers on the subject of growth factors have raised the question whether these organisms are totally unable to synthesize the growth factors or whether they are unable to synthesize enough of the factor for rapid multiplication during the period of rapid development of the culture. This question can be answered for the dysentery bacilli by an examination of the data presented in table 4 and figure 1. It will be seen that at low concentra-

tions of nicotinamide (0.001–0.01 micrograms per cc. of medium) there is a limit beyond which a culture will not develop, whether measured by turbidity or by acid produced. This would indicate that below a certain concentration the amount of cellular division that can occur is limited by the amount of nicotinamide that is available.

Synthesis of coenzymes by dysentery bacilli.—The next question is whether nicotinamide is used by the dysentery bacillus for synthesis of one

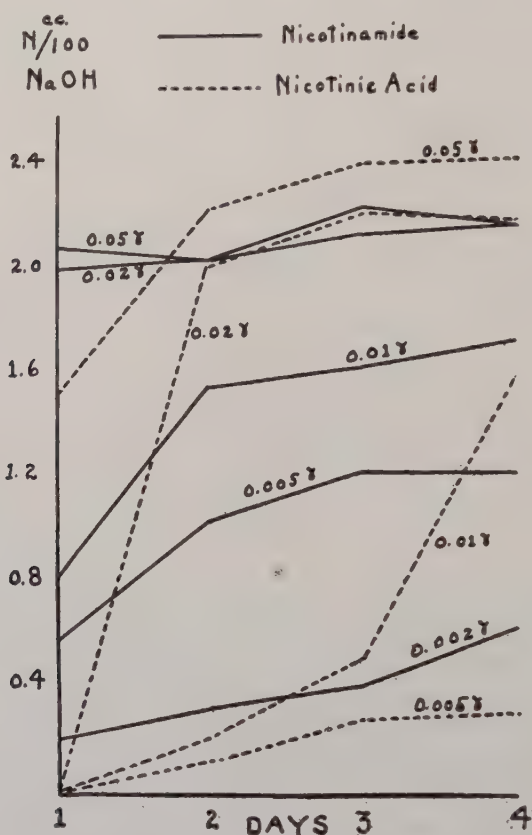


Fig. 1.—Titration plotted against concentration of nicotinic acid and nicotinamide.

of the coenzymes. Accordingly, tests for coenzymes were made on the filtrates of dysentery cultures after development in the nicotinic acid-synthetic medium. By use of *Hemophilus influenzae* or *Hemophilus parainfluenzae* a method is afforded for the detection of the coenzymes, since Lwoff and Lwoff²³ have shown that the coenzymes will replace the "V" factor needed by these organisms for development in nutrient broth. Furthermore, components of the coenzyme molecule such as nicotinic acid and nicotinamide failed to take the place of the intact molecule in satisfying the growth requirements of *Hemophilus*. If coenzyme is synthesized by dysentery bacilli, filtrates of

23. Proc. Roy. Soc., London, s. B. 122: 352, 1937.

dysentery cultures grown in a synthetic medium should support growth of the *Hemophilus* organisms when added to a broth medium containing hemin.

Cultures were started in the usual synthetic medium with 0.5 microgram of nicotinic acid per cc. of medium. At the end of 3 days the culture was frozen in an acetone-solid carbon dioxide bath. After 10 minutes immersion in the freezing bath the tubes were thawed in hot water. The freezing and thawing were repeated 5 times. The cultures were then filtered through a sintered glass filter. Each filtrate was tested for sterility by inoculation of a small amount into veal infusion broth.

Various quantities of filtrate were added to tubes of dextrose veal infusion broth. The tubes to be inoculated with *Hemophilus influenzae* received also 1 microgram per cc. of hemin, since this species, in contrast to *Hemophilus parainfluenzae*, requires hemin in addition to the "V" factor. With each test control tubes were included: (a) the dextrose veal infusion broth; (b) broth with the addition of the uninoculated nicotinic acid-synthetic medium; (c) broth with cozymase and hemin and (d) broth with addition of sheep blood which serves as a source of both coenzyme and hemin.

TABLE 5.—*Effect of Dysentery Bacillus Filtrates Upon Development of Hemophilus Species*

5 cc. Tubes of Dextrose Veal Infusion Broth,* plus:	H. influenzae, Strain 600		H. parainfluenzae, Strain 429	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1.0 cc. filtrate, dysentery 76	++	++	+++	+++
0.5 cc. filtrate, dysentery 76†	++	++	+++	+++
0.2 cc. filtrate, dysentery 76	++	++	++	+++
0.1 cc. filtrate, dysentery 76	+	+	+	+
Controls:				
1.0 cc. synthetic medium containing nicotinic acid	0	0	0	0
Coenzyme I, 1.0 microgram per cc.	++	++	+++	+++
10% sheep blood	++	++	+++	+++
Nothing added to the broth	0	0	0	0

* 1 microgram per cc. of hemin was included when *H. influenzae* was the test organism.

† The volume of all tubes was made equal by the addition of an appropriate amount of 0.9% sodium chloride solution.

Results obtained on the filtrate of one dysentery culture are summarized in table 5. In this experiment one strain of *Hemophilus influenzae* and one of *Hemophilus parainfluenzae* were used. In other experiments different strains of dysentery bacilli and of *Hemophilus* were used with similar results. The evidence indicates that nicotinic acid may be used by dysentery bacilli for synthesis of coenzymes. Since both cozymase and coenzyme II are able to promote growth of *Hemophilus*, it is impossible to state which of these compounds has been synthesized. It is also possible that there is formed by the dysentery bacilli some still unknown compound which is able to support growth of the *hemophilus* species.

Synthesis of growth factors for staphylococcus.—The same filtrates of dysentery bacillus cultures were added to the usual synthetic medium and the medium then inoculated with *staphylococcus*. Knight¹¹ has shown that both nicotinic acid (or the amide) and thiamin are required by *Staphylococcus aureus* and also that the two component ring structures of thiamin will suffice instead of the intact thiamin molecule. In the synthetic medium used in our work several strains of *staphylococci* were able to develop upon addi-

tion of nicotinic acid and thiamin, though growth was often considerably slower than that secured in broth. Development of staphylococcus in the synthetic medium after substituting a dysentery bacillus filtrate for thiamin would indicate a synthesis of the two ring structures of thiamin or perhaps of one or more unknown factors needed by this organism.

The results of a typical experiment are given in table 6. Growth of the *Staphylococcus albus* culture occurred after addition of dysentery bacillus synthetic medium filtrates. The growth under these conditions was considerably slower than in veal infusion broth, but also slightly more rapid than that occurring in the presence of nicotinamide and thiamin. The strain of *Staphylococcus aureus* shows several interesting features. This culture upon repeated tests in the synthetic medium with added nicotinamide and thiamin either refused to grow or at times grew very slowly. It developed readily, however, in the usual-veal infusion broth. Evidently one or more unknown stimulators are needed by this strain and are supplied to some extent by the dysentery filtrates.

TABLE 6.—*Effect of Dysentery Bacillus Filtrates Upon Development of Staphylococcus*

5 cc. Tubes of Synthetic Medium Containing 1.0 Microgram per cc. of Nicotinamide, Plus One of the Following:	Staphylococcus albus Laboratory Stock				Staphylococcus aureus Strain A			
	1	Growth, Days			1	Growth, Days		
		2	3	4		2	3	4
1.0 cc. filtrate, dysentery 76	0	+	+++	+++	0	++	+++	+++
0.2 cc. filtrate, dysentery	0	0	0	0	0	++	+++	+++
Controls:								
Synthetic medium only	0	0	0	0	0	0	0	0
Thiamin, 1 microgram per cc.	0	0	++	+++	0	0	0	0
Veal infusion broth	++++				++++			

The filtrate of dysentery 76 was prepared from a culture grown in the synthetic medium plus 0.5 microgram per cc. of nicotinic acid.

Our results show that something needed by the staphylococcus is produced by dysentery bacilli when growing in the synthetic medium. It seems probable that both thiamin (or its two component ring structures) and at least one other factor are synthesized. It is possible that the other factor may be biotin, for Kögl and van Wagtenonk²⁴ have reported that biotin stimulated growth of staphylococcus, particularly when added along with nicotinic acid and thiamin.

Synthesis of riboflavin.—Riboflavin-phosphoric acid is known to be the prosthetic group of a yellow enzyme which is capable of oxidizing the reduced form of either of the known pyridine nucleotides. This fact together with the fact that the dysentery bacilli do not require riboflavin for growth, made it of special interest to determine whether these organisms, when growing in a synthetic medium, synthesize riboflavin. We are deeply indebted to Dr. E. E. Snell of the University of Wisconsin for his aid in carrying out this test.

Four different strains of dysentery bacilli were used in this study. The cultures were grown on the previously described synthetic medium containing 0.1 microgram of nicotinamide per cc. of medium. At the end of three days

24. Rec. d. trav. chim. d. Pays-Bas 57: 747, 1938.

incubation the cultures were autoclaved and the whole culture was assayed for riboflavin by Dr. Snell, using a method depending on the growth-promoting properties of riboflavin for a strain of lactic acid bacteria. Values ranging from 0.175 microgram to 0.130 microgram for a 5 cc. culture were obtained. No riboflavin was present in the basal medium. All cultures tested synthesized some riboflavin, but there was a quantitative variation among strains.

This would indicate that these organisms which are unable to synthesize nicotinamide are able to synthesize riboflavin.

THE EFFICACY OF CERTAIN COMPOUNDS RELATED TO NICOTINAMIDE AS GROWTH FACTORS FOR DYSENTERY BACILLI

The next step in our investigation was a comparative study of the efficacy of pyridine derivatives. If nicotinic acid plays a role in the bacterial cell similar to that played in the mammalian organism there should be a close correspondence between the two in the response to various compounds which are chemically related to nicotinic acid. In applying these compounds to the study of bacteria it is possible to secure readily quantitative data which have been difficult or impossible to obtain in the study of canine blacktongue. One difficulty encountered in our work was the great sensitivity of the bacteria; the micro-organisms react to impurities in the compounds which escape recognition by the usual chemical methods.

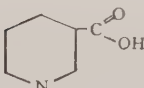
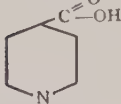
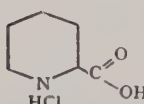
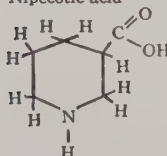
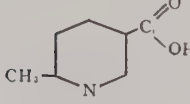
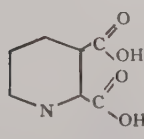
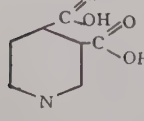
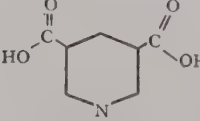
In comparing the growth response of the dysentery bacilli with the curative effect on canine blacktongue we were particularly fortunate, since we were able to assay samples of the same preparations of some compounds which were used by Woolley, Strong and coworkers²⁵ in their work on blacktongue. The following compounds were obtained from Dr. Strong: isonicotinic acid, nipecotic acid, 6-methyl nicotinic acid, β -acetyl pyridine, ethyl nicotinoacetate, nicotinic acid N-methylamide, nicotinuric acid and trigonelline.

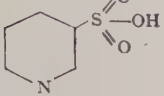
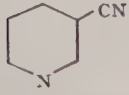
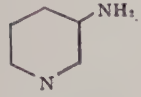
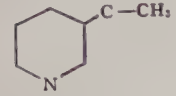
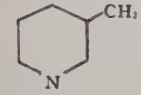
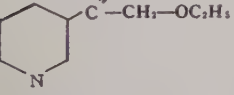
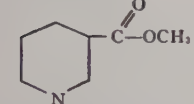
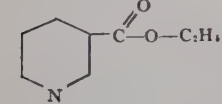
In all we have assayed 24 compounds related to nicotinic acid. The compounds were prepared by standard methods and were identified by physical constants in the literature. In 2 cases they were identified by analyses. All compounds were made up in 1×10^{-3} molar solutions and were sterilized by filtration through glass filters. The original solution was diluted so that small amounts, when added to 5 cc. of the synthetic medium, produced a graded series of concentrations.

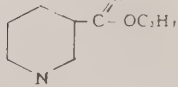
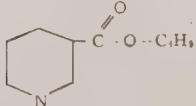
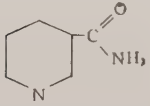
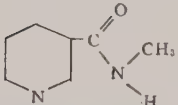
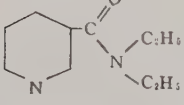
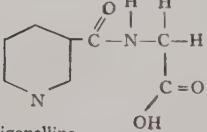
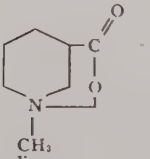
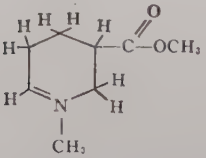
Table 7 shows the composite results obtained on 3 strains of *Shigella paradysenteriae*, Flexner. It will be noted that the 2 isomers of nicotinic acid, picolonic acid and isonicotinic acid, are completely devoid of activity. In this connection it is interesting to note some of the difficulties encountered with picolonic acid. A sample of picolonic acid obtained from Dr. Strong was found to support growth at a concentration of 1×10^{-4} M. This was disturbing since he had found the same preparation completely inactive for blacktongue. However a sample prepared from β -picoline which had been purified through the mercury salt was totally inactive.

25. J. Biol. Chem. **124**: 715, 1938.

TABLE 7.—*Activity of Pyridine Derivatives as Growth Factors for Dysentery Bacilli and Comparison with Other Activities*

Compound	Development of Dysentery Bacilli in a Synthetic Medium*					Other activities reported in literature		
	Molar Concentration	1	2 Days	4	7	Staph. aureus ^{22, 26}	Black-tongue ^{25, 27}	Pellagra ²⁸
Nicotinic acid 	1×10^{-4} 3×10^{-7} 1×10^{-7} 3×10^{-8}	+++ 0 0 0	++++ ++++ 0 0	++++ ++++ ++ 0	++++ ++++ ++++ ++	K ⁺ 1×10^{-4} L ⁺ 2×10^{-4}	Rapid improvement	Rapid improvement
Isonicotinic acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0	Inactive	Dog grew worse	
Picolinic acid—HCl 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0	Inactive	Dog grew worse	
Nipecotic acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0		No improvement	
6-Methyl nicotinic acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0		No improvement	
Quinolinic acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	++ 0 0	+++ 0 0	+++ 0 0	L Inactive		
Cinchomeric acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0			
Dinicotinic acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0			

Compound	Development of Dysentery Bacilli in a Synthetic Medium*					Other activities reported in the literature		
	Molar Concentration	1	2	Days 4	7	Staph. aureus ^{22,26}	Black-tongue ^{25,27}	Pellagra ²⁸
Pyridine 3-sulfonic acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0		Dog dead in 3 days Dog dead in 2 days	
Nicotino-nitrile 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0	K Inactive	No improvement	
β -Amino pyridine 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0		No improvement	
β -Acetyl pyridine 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0		Dog grew worse	
β -picoline 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0	K Inactive	Rapid improvement	
Ethyl nicotino acetate 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0		Dog grew worse	
Pyridine 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0			
Methyl nicotinate 	1×10^{-6} 1×10^{-7} 1×10^{-8}	+++ +++ 0	+++ +++ 0	+++ +++ 0	+++ +++ ++	5×10^{-7} K		
Ethyl nicotinate 	1×10^{-6} 1×10^{-7} 1×10^{-8}	+++ +++ 0	+++ +++ 0	+++ +++ +	+++ +++ +++	2×10^{-6} L	Rapid improvement	

Compound	Development of Dysentery Bacilli in a Synthetic Medium*					Other Activities Reported in the literature		
	Molar Concentration	1	2	4	7	Staph. aureus ^{22,28}	Black-tongue ^{26,27}	Pellagra ²⁸
Propyl nicotinate 	1×10^{-4} 1×10^{-5} 1×10^{-6}	++ 0 0	+++ ++ 0	+++ +++ +++	+++ +++ +++			
Butyl nicotinate 	1×10^{-4} 1×10^{-5} 1×10^{-6}	++ 0 0	+++ ++ 0	+++ +++ ++	+++ +++ +++			
Nicotinamide 	1×10^{-7} 3×10^{-8} 1×10^{-8}	+++ +++ +	+++ +++ +	+++ +++ +	+++ +++ +	K 2×10^{-7} L 1×10^{-6}	Rapid improvement	Active
Nicotinic acid N-methyl amide 	1×10^{-4} 1×10^{-5} 1×10^{-6}	+++ ++ 0	+++ +++ 0	+++ +++ 0	+++ +++ 0		Rapid improvement	
Coramine 	1×10^{-4} 1×10^{-5} 1×10^{-6}	+++ 0 0	+++ 0 0	+++ 0 0	+++ ++ 0	K Inactive L Inactive		Active
Nicotinuric acid 	1×10^{-4} 1×10^{-5} 1×10^{-6}	+++ +++ +	+++ +++ ++	+++ +++ ++	+++ +++ +++	L 2×10^{-6}	Rapid improvement	
Trigonelline 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0	K Inactive L Inactive	No improvement	
Arecoline 	1×10^{-4} 1×10^{-5} 1×10^{-6}	0 0 0	0 0 0	0 0 0	0 0 0			

* Results based on three strains of *Shigella paradysenteriae*.

† K refers to results reported by Knight.²²

‡ L refers to results reported by Landy.²⁹

26. Landy, M.: Proc. Soc. Exper. Biol. & Med. **38**: 504, 1938.

27. Strong, F. M., Madden, R. J., and Elvehjem, C. A., J. Am. Chem. Soc. **60**: 2565, 1938.

28. Spies, T. D., Bean, W. B., and Stone, R. E.: J.A.M.A. **111**: 584, 1938.

Nipecotic acid was found to be completely devoid of activity. Thus complete reduction of the ring results in total loss of activity. The substitution of a methyl group in the ring results in complete loss of activity.

Three dicarboxylic acids of pyridine, quinolinic, cinchomeric and dinicotinic, were assayed. Quinolinic acid was weakly active but the others were inactive. The weak activity of quinolinic acid might be due to a partial decarboxylation, resulting in the formation of nicotinic acid.

β -amino pyridine was completely inactive. This compound was of interest since Subbarow and Dann²⁹ have reported that it could be isolated from liver and was capable of curing blacktongue. They later retracted this statement,³⁰ and at the same time Strong and coworkers²⁷ reported it to be inactive.

Both pyridine and β -picoline were completely inactive. β -picoline compound should cure blacktongue but not support growth of the dysentery organism is not surprising, since it has been shown by Sendju³¹ that when α -picoline is fed to dogs it is oxidized to picolinic acid.

The methyl, ethyl, propyl and butyl esters of nicotinic acid were assayed. The methyl ester was somewhat more active than the acid. This increase in activity may be correlated with the fact that the methyl ester is converted in vitro to the amide more readily than is the acid. The butyl ester, which was prepared according to Engler's method³² has not been reported in the literature. It boils at 237 C.³³

Substitution of one methyl group on the amide nitrogen of nicotinamide decreases the activity and substitution of two methyl groups still further reduces the activity.

Nicotinuric acid was found to have an activity comparable to that of nicotinic acid. This compound is a normal excretion product of the dog. The activity of this compound is an important consideration in the determination of the nicotinic acid content of urine by biological methods.

Trigonelline is a betaine of nicotinic acid which occurs naturally in urine.³⁴ It has been found to be entirely inactive and is completely ineffective in the cure of blacktongue.²⁷ Arecoline, a naturally occurring alkaloid related to nicotinic acid, is without activity, which is not surprising since the ring nitrogen is blocked making the linkage to ribose in the coenzyme impossible. This compound was obtained from Dr. Gladstone of the Department of Chemistry of the University of Chicago.

Table 7 shows also a comparison of activity of various derivatives which have been tried on *Staphylococcus aureus*, dysentery bacilli, pellagra and blacktongue. With a few exceptions, the results obtained by various workers have been consistent.

We found that coramine was able to support growth of the dysentery bacillus while both Knight and Landy found this compound was unable to support growth of *Staphylococcus aureus*. This disagreement might be ex-

29. J. Am. Chem. Soc. **60**: 1510, 1938.

30. J. Am. Chem. Soc. **60**: 2565, 1938.

31. J. Biochem. **7**: 273, 1927.

32. Ber. d. deutsche chem. Gesellschaft., **27**: 1784, 1894.

33. Difficulty was encountered in obtaining a nitrogen analysis, after repeated high vacuum distillations, which agreed closer than one half per cent with the calculated value.

34. Kutscher, and Lohman: Ztschr. f. physiol. Chem. **49**: 85, 1906.

plained by the fact that these investigators did not use as large concentrations as we did. Our results with smaller amounts agree more closely with their findings.

The similarity of the results obtained in the various studies on nicotinic acid derivatives indicates that a similarity in the mode of action of these compounds may exist.

SUMMARY

Nicotinic acid was isolated from pig spleen and the presence of the amide was strongly indicated. The growth-promoting activity of spleen extracts for dysentery bacilli can be attributed to the presence of either or both of these compounds.

Other known growth factors, with the exception of coenzymes I and II, were not capable of replacing either partially or completely nicotinic acid or its amide.

Thirty-three representative cultures of the *Shigella* group were tested for their ability to develop in a synthetic medium alone and with the addition of nicotinic acid, nicotinamide, coenzyme I or coenzyme II. Many of the cultures were unable to grow in the synthetic medium but developed readily upon the addition of small amounts of either the coenzymes, nicotinic acid or its amide.

Dysentery cultures which required nicotinic acid or the amide were able to develop through successive transfers when these compounds were supplied in a synthetic medium.

Amounts of nicotinic acid up to 1,000 micrograms per cc. of medium appeared to exert no restraining effect. With 3,000, 5,000 and 10,000 micrograms per cc. a progressive inhibition was found.

Nicotinamide was at least ten times as potent as nicotinic acid when cultural development was compared 24 hours after inoculation. With longer periods of incubation the difference in effect of the two compounds was less marked. This was due to delayed development of the culture when limited amounts of nicotinic acid were supplied and may indicate conversion of the acid to the amide before utilization.

The dysentery bacillus growing in a synthetic-nicotinic acid medium synthesized a substance capable of promoting growth of several *Hemophilus* species, presumably coenzyme I or II or some related substance.

The dysentery bacillus synthesized growth essentials required by certain strains of staphylococci. The essentials may be thiamin or its component parts and possibly biotin. This bacillus also synthesized riboflavin.

The activity of 24 compounds structurally related to nicotinic acid was studied. Any change in ring substitution resulted in loss of activity. Esterification resulted in decreasing activity with increasing length of the alkyl chain. Substitution on the amide nitrogen resulted in a marked decrease in activity. Nicotinuric acid was comparable in activity to nicotinic acid.

A close correspondence was found between the activity of these compounds as essential growth factors for the dysentery bacillus, the reported activity for *Staphylococcus aureus* and the curative effect on canine black-tongue and human pellagra.

